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Effect of miR-7 on resistance of breast cancer cells to adriamycin *via* regulating EGFR/PI3K signaling pathway

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Abstract. – OBJECTIVE: To explore whether micro ribonucleic acid (miR)-7 affects the resistance of breast cancer cells to adriamycin (ADR) through regulating the epidermal growth factor receptor (EGFR)/phosphatidylinositol 3-hydroxy kinase (PI3K) signaling pathway.

MATERIALS AND METHODS: MiR-7 expression was compared among MCF-10A, MCF-7 and MCF-7/ADR cells. The MCF-7/ADR cells were divided into three groups, namely miR-7 control group (MCF-7/ADR drug-resistant strains), miR-7 inhibition group (miR-7-inhibited MCF-7/ADR drug-resistant strains) and miR-7 promotion group (MCF-7/ADR drug-resistant strains transfected with miR-7), and the messenger RNA (mRNA) and protein expression levels of MCF-7/ADR were evaluated via Western blotting.

RESULTS: The expression level of miR-7 was substantially decreased in MCF-7 and MCF-7/ADR cells ($p<0.05$), and it was lowered more obviously in MCF-7/ADR cells than that in MCF-7 cells ($p<0.05$). Compared with that in miR-7 control group, miR-7 expression in miR-7 promotion group was notably raised ($p<0.05$), proving that the sensitivity of MCF-7/ADR cells to ADR was enhanced, while that in miR-7 inhibition group was significantly lowered ($p<0.05$). Compared with those in miR-7 control group, the mRNA and protein expression levels of EGFR and PI3K were elevated in miR-7 inhibition group ($p<0.05$), while they were lowered in miR-7 promotion group ($p<0.05$). Additionally, compared with those in miR-7 control group, the proliferation and apoptosis abilities of cells in miR-7 inhibition group were markedly enhanced ($p<0.05$) and weakened ($p<0.05$), respectively, while they were weakened ($p<0.05$) and significantly strengthened ($p<0.05$), respectively in miR-7 inhibition group.

CONCLUSIONS: MiR-7 plays an important role in the resistance of breast cancer cells to ADR, and its over-expression can inhibit the EGFR/PI3K signaling pathway to raise their sensitivity to the chemotherapy drug ADR.

Key Words:
EGFR, PI3K, Breast cancer, Resistance to Adriamycin, miR-7.

Introduction

The prevalence rate of breast cancer is increasingly higher among women, and according to the survey of the World Health Organization, the cases of this disease represents about 30% of the total in women worldwide[1]. Generally, the affected population is dominated by middle-aged women, and the incidence rate is gradually decreasing after the age of 55 years old[2]. However, the affected population of breast cancer is gradually getting younger, and the malignancy of tumor is also higher. Additionally, this tumor is related to family heredity history to some extent[3,4]. As patients are normally treated via radical resection and chemotherapy, the clinical emphasis is to repress the occurrence and development of breast cancer. Micro ribonucleic acid-7 (miR-7) is a crucial regulatory factor that affects tumor cell migration and invasion in some cancers, like gastric cancer[5]. Some studies have proven that miR-7 can regulate the expression of functional proteins to play important roles in various physiopathological processes in breast cancer[6-8], but

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whether miR-7 is able to affect the resistance of breast cancer cells to Adriamycin (ADR) via the regulation of the epidermal growth factor receptor (EGFR)/phosphatidylinositol 3-hydroxy kinase (PI3K) signaling pathway remains to be explored. EGFR, an epidermal growth factor, is involved in the signaling pathways and can promote cell proliferation and transduction. The over-expression of EGFR plays an extraordinary role in the occurrence and development of malignancies, and it is highly expressed in tumors[9]. PI3K is able to suppress and activate cells. LeBlanc et al[10] confirmed that regulating the expression of miR-7 can reverse the resistance of breast cancer cells to ADR by the possible mechanism that miR-7 regulates the expression of ABCC10. Piro et al[11] have corroborated that miR-1268b inhibits the resistance of breast cancer cells to chemotherapy drugs via down-regulating the EGFR/PI3K signaling pathway[12]. The present study explored the influence of miR-7 on the resistance of breast cancer cells to ADR via the regulation of the EGFR/PI3K signaling pathway, providing a reliable basis for the chemotherapy resistance of breast cancer in clinic.

Materials and Methods

Materials and Reagents

Normal breast cells (MCF-10A) were purchased from Hanbio Biotechnology Co., Ltd. (Shanghai, China), MCF-7/ADR drug-resistant breast cancer cell strains and MCF-7 human breast cancer cell strains from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), ADR from Beyotime Biotechnology (Shanghai, China), dye reagent from Dalian TaKaRa (Otsu, Shiga, China), radioimmunoprecipitation assay (RIPA) lysate and CCK-8 from Solarbio (Beijing, China), PI3K inhibitor LY294002 from Beyotime Biotechnology (Shanghai, China), mouse anti-human β-actin monoclonal antibody from Promega (Madison, WI, USA), rabbit anti-human RAB2A polyclonal antibody from Bowest (Nuaillé, France), rabbit anti-human phosphorylated protein kinase B (p-Akt)-473 polyclonal antibody from Beijing ComWin Biotech Co., Ltd. (Beijing, China), rabbit anti-human p-Akt-308 polyclonal antibody from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China) and fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) from Beyotime Biotechnology (Shanghai, China).

Instruments

ABI7500 fluorescence quantitative polymerase chain reaction (PCR) instrument was purchased from Applied Biosystems (Foster City, CA, USA), electrophoresis apparatus and transfer instrument from ABI (Foster City, CA, USA), CO_2 incubator from Shanghai Sunny Hengping Scientific Instrument Co., Ltd. (Shanghai, China), and microplate reader from Shanghai Anting (Shanghai, China).

Cell Transfection

MCF-10A, MCF-7 and MCF-7/ADR cells were cultured in a DMEM containing 10% FBS and 1% double antibodies, separately, and the medium was replaced once every other day. After culture, the MCF-10A and MCF cells were directly used for miR-7 expression. The MCF-7/ADR cell strains were applied to experiments and divided into miR-7 control group, miR-7 inhibition group and miR-7 promotion group. In miR-7 control group, MCF-7/ADR cells were routinely cultured only with liposomes and without any other treatment. In miR-7 inhibition group, the cells were added with liposomes and transfected with control plasmids, while those in miR-7 promotion group were transfected with miR-7 plasmids with the sequence of 5'-JUGUUUUAGUGAUCAAGAAGGU-3', and added with 1.0 mg/L ADR to maintain their resistance to the drug.

Reverse Transcription (RT)-PCR

MCF-7/ADR cell strains were digested with trypsin, cooled and washed using phosphate buffered saline (PBS) once. With the supernatant discarded, the cell strains were added with 100 μL of trichloromethane and 2 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), fully stirred for evenly mixing and let stand for 5 min, and then the mixture was centrifuged at 13000 rpm and 5°C for 5 min. The MCF-7/ADR cell tissues were made to three layers, and the upper water phase was transferred into a 1.5 mL Eppendorf (EP) tube and added with isopropanol to prepare the reaction solution. The RNAs were extracted from the samples and reversely transcribed into cDNAs. The PCR conditions are as follows: 94°C for 40 min, 60°C for 35 min, 72°C for 30 s and 90°C for 3 min, for a total of 45 cycles. Primer sequences are shown in Table I.

Table I. Primer sequence.

Gene		Primer sequence
MiR-7	Forward	5'-AAAAGAACACGTGGAAGGATAG-3'
	Reverse	5'-CGCCTAACGTACCGCGAATTT-3'
EGFR	Forward	5'-AAGTGTGGCTCCAGGAGTATC-3'
	Reverse	5'-ACTGTCAGATTGCACGGTGTC-3'
PI3K	Forward	5'-ACATCATAATCGGCGACACAGGTG-3'
	Reverse	5'-ATTCGAGCACCGAACTCTACACC-3'
GAPDH	Forward	5'-ATCACTGCCACTCAGAAG-3'
	Reverse	5'-AAGTCACAGGAGACAACC-3'

Immunohistochemical Detection of Proteins

The EGFR and PI3K proteins in the breast cancer drug-resistant cell strains were detected. The cell tissues were firstly fixed in 5% paraformaldehyde overnight, dehydrated, embedded into the paraffin and serially sectioned into blocks (2×2×2 μm in length × width × height). After induction for 24 h or 48 h, the MCF-7/ADR cell strains were set as experiment groups. The collagen in the three groups of MCF-7/ADR cell strains was observed via Masson's trichrome staining and deposited. Then the protein expressions of EGFR and PI3K were detected using HE staining and WGA staining. After antigen retrieval, the tissue sections were sealed using serum for 20 min and incubated with the secondary antibody using the two-step method for 50 min, followed by color development using diaminobenzidine. Finally, the protein expressions of EGFR and PI3K were observed under a light microscope.

Western Blotting

MCF-7/ADR cells were inoculated into a 6-well plate, digested with 100 μg of trypsin extract and then added with 1% PMSF. The cell extract was placed in an EP tube, mixed with trypsin extract at 1:100 and fully lysed using RIPA lysate. Subsequently, the resulting products were transferred onto membranes, sealed in 5% skim milk powder and incubated with the primary antibodies at 40°C overnight. After washing, the membranes were incubated with the secondary antibodies and washed again, followed by addition of electrochemiluminescence (ECL) for exposure. Finally, EGFR (1:500) and PI3K (1:1000) were analyzed using the Quantity One software.

Detection of Cell Proliferation via Methyl Thiazolyl Tetrazolium (MTT) Assay

After being transfected for 24 h, MCF-7/ADR cell strains were digested with trypsin and seeded into a 96-well plate. In the three groups, 5 wells were set at each time point. After inoculation, cells were taken out from three 96-well plates and added with 20 μL of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) for detection. Next, the three groups of cells were cultured for another 2 h, added with 10 μL of ADR and continued to be cultured for 24, 48, 72 and 96 h. Finally, the absorbance of cells was measured at 45 nm using the microplate reader.

Detection of Cell Apoptosis via Flow Cytometry

MCF-7/ADR cell strains with the density adjusted to 1×10^8 cells/L were inoculated into a 6-well plate. Then, each well (3 mL in volume) was added with the equal volume of ADR at the final concentrations of 0, 25, 50, 100 and 200 μmol/L, respectively. Subsequently, the cells were cultured in the CO_2 incubator for 48 h. All the groups of cells were harvested and centrifuged at a radius of 5 cm for 10 min. With the supernatant discarded, the cells were washed using PBS twice and re-suspended in 500 μL of PBS. Finally, the apoptosis of cells was detected as specified in the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit.

Statistical Analysis

Results were obtained through at least three independent experiments and expressed as the mean ± standard deviation. The t-test was used for analyzing measurement data. A comparison between multiple groups was done using One way ANOVA test followed by Post-Hoc Test LSD (Least Significant Difference). The protein and messenger RNA (mRNA) expressions of miR-7 in miR-7 control group, miR-7 inhibition group and miR-7 promotion group were detected, and all data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). $p<0.05$ was considered as statistically significant.

Results

Difference in miR-7 Expression Among MCF-10A, MCF-7 and MCF-7/ADR Cells

It was found through RT-PCR that the expression level of miR-7 was substantially decreased in human MCF-7 and MCF-7/ADR cells ($p<0.05$), and the decrease was more evident in MCF-7/ADR cells than that in MCF-7 cells ($p<0.05$), suggesting that the expression level of miR-7 is decreased in breast cancer cells and lower in ADR-resistant ones (Figure 1).

MiR-7 Expression in MCF-7/ADR After Transfection

According to the MTT results, after transfection, miR-7 promotion group had a notably raised expression level of miR-7 than miR-7 control group ($p<0.05$), proving that the sensitivity of MCF-7/ADR cells to ADR was enhanced. However, compared with that in miR-7 control group, the expression level of miR-7 in miR-7 inhibition group was significantly decreased ($p<0.05$), indicating that once miR-7 is inhibited, the sensitivity to ADR has also weakened accordingly (Figure 2).

Expressions of mRNAs Related to the EGFR/PI3K Signaling Pathway in MCF-7/ADR

The quantitative RT-PCR results indicated the relationship between the mRNA expression of miR-7 and that of EGFR and PI3K in MCF-7/ADR, namely when the mRNA expression of miR-7 was raised, the one of EGFR and PI3K was decreased, and when it was lowered, the latter was elevated. Compared with those in miR-7 control group, the mRNA expression levels of EGFR and PI3K in miR-7 inhibition group were notably raised ($p<0.05$), while they were lowered in miR-7 promotion group ($p<0.05$) (Table II).

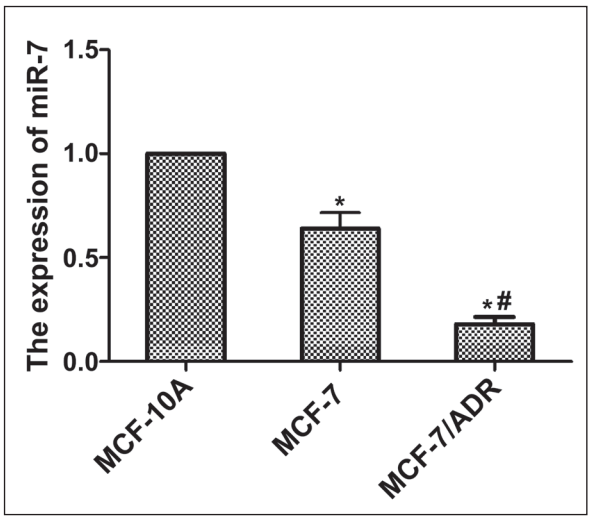

Figure 1. Expression of miR-7 in three groups of cell strains. Note: *: $p<0.05$, *vs.* MCF-10A, and #: $p<0.05$, *vs.* MCF-7.

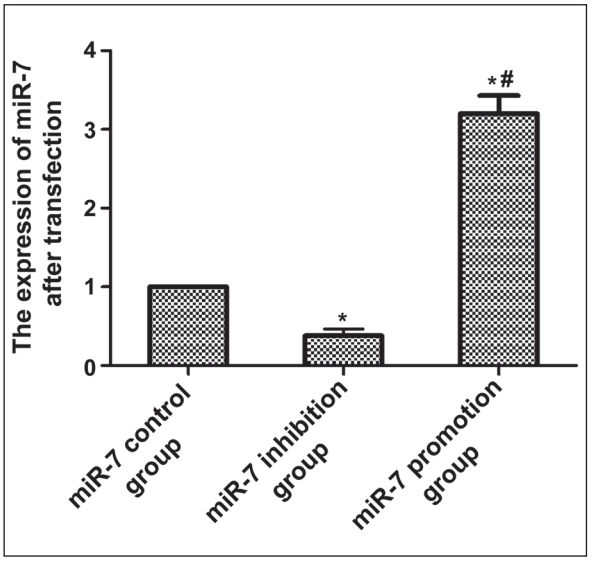

Figure 2. Expression of miR-7 in MCF-7/ADR after transfection. Note: *: $p<0.05$, vs. miR-7 control group, and #: $p<0.05$, vs. miR-7 inhibition group.

Protein Expression of EGFR/PI3K Signaling Pathway in MCF-7/ADR

The expression levels of EGFR/PI3K signaling pathway proteins in MCF-7/ADR in the three groups were determined via immunohistochemistry. It was found that compared with those in miR-7 control group, the expression levels of EGFR and PI3K were substantially raised in miR-7 inhibition group ($p<0.05$), while they were lowered in miR-7 promotion group ($p<0.05$) (Figure 3-5).

Relationship Between miR-7 and MCF-7/ADR Cell Proliferation

In the MTT assay, it was found that over-expressing miR-7 affected the proliferation ability of MCF-7/ADR cells. After action by ADR for 48 h, the proliferation ability of cells in the three groups was enhanced. After action for 72 h, it was stable. Compared with that in miR-7 control group, the cell proliferation ability was notably

Table II. MRNA expression levels of EGFR and PI3K in MCF-7/ADR.

Group	MiR-7 mRNA	EGFR mRNA	PI3K mRNA
MiR-7 promotion group	2.41 ± 0.16*#	0.72 ± 0.09*#	0.99 ± 0.09*#
MiR-7 control group	0.67 ± 0.13	2.31 ± 0.11	1.87 ± 0.14
MiR-7 inhibition group	0.43 ± 0.09*#	2.43 ± 0.14*#	1.98 ± 0.13*#

Note: *: $p<0.05$, *vs*. miR-7 control group, and #: $p<0.05$, *vs*. miR-7 inhibition group.

strengthened in miR-7 inhibition group ($p<0.05$), but weakened in miR-7 promotion group ($p<0.05$) (Figure 6).

Relationship Between miR-7 and MCF-7/ADR Cell Apoptosis

The influence of miR-7 on the apoptosis ability of MCF-ADR cells was detected via flow cytometry, and it was discovered that compared with that in miR-7 control group, the cell apoptosis ability was notably weakened in miR-7 inhibition group ($p<0.05$), but enhanced in miR-7 promotion group ($p<0.05$) (Figure 7).

Discussion

Among breast cancer patients, the proportion of women to men is as high as 1:9[14]. Breast cancer is partly associated with hereditary factors and partly caused by improper underwear, drinking, high-fat diet, and anxiety. According to statistics, there are about 900 thousand new cases of breast cancer every year in China, and the patients are younger, so it is urgent to seek effective treatment

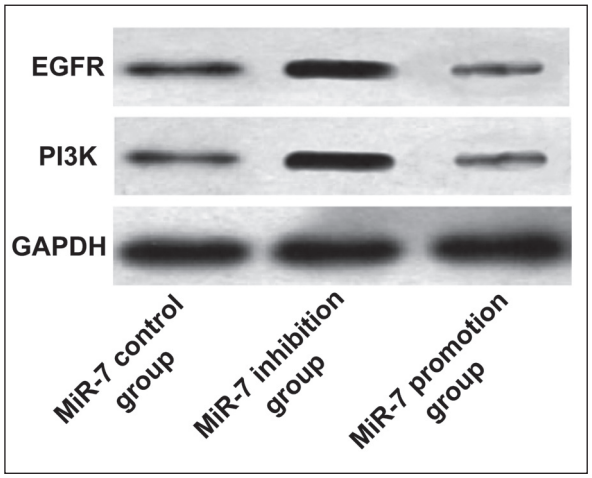

Figure 3. Protein expressions of EGFR and PI3K.

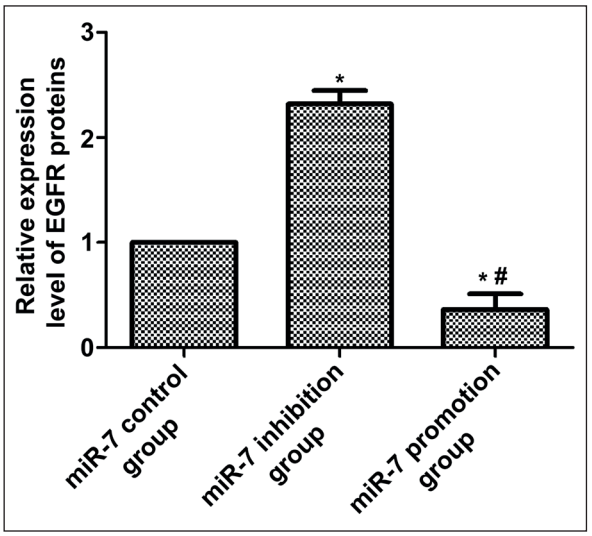

Figure 4. Relative expression level of EGFR proteins. Note: *: $p<0.05$, *vs*. miR-7 control group, and #: $p<0.05$, *vs*. miR-7 inhibition group.

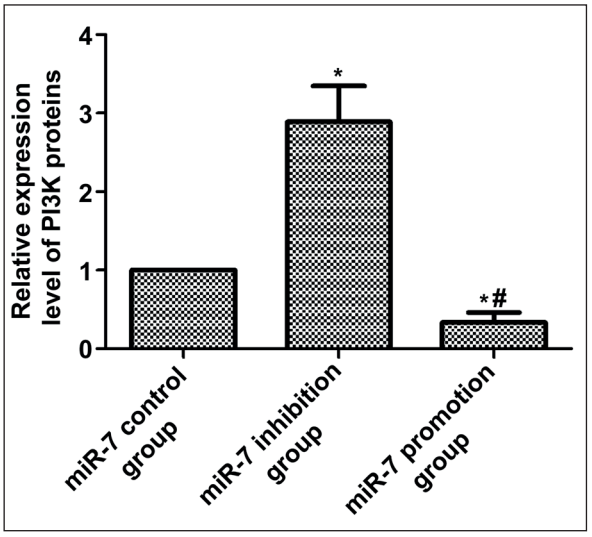

Figure 5. Relative expression level of PI3K proteins. Note: *: $p<0.05$, *vs*. miR-7 control group, and #: $p<0.05$, *vs*. miR-7 inhibition group.

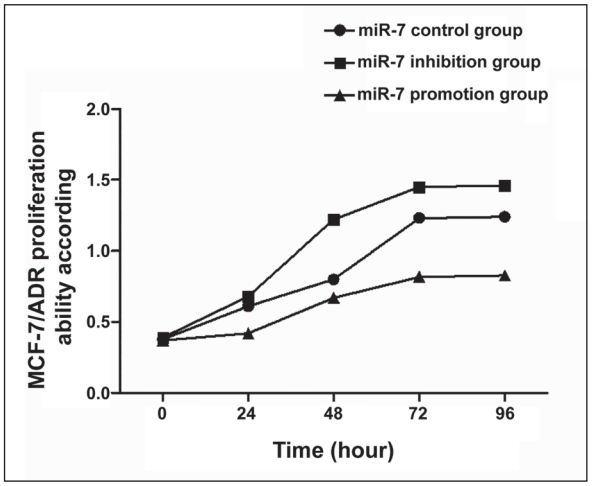

Figure 6. MCF-7/ADR proliferation ability according to the absorbance at 450 nm. Note: After action by ADR for 48 h, the proliferation ability of cells is enhanced in the three groups. After action for 72 h, the cell proliferation ability is stable, and it is weaker in miR-7 promotion group than that in miR-7 control group and miR-7 inhibition group.

and cure means for breast cancer. With the rapid development of medical technologies, breast cancer has been no longer treated using a single approach, and the treatments have continued to be shifted from conventional resection to chemotherapy and radiotherapy which are combined to play crucial roles in preventing the recurrence and metastasis of breast cancer, improving the survival quality of patients and prolonging their life[15-17]. As one of typical medications in chemotherapy for breast cancer, ADR has significant therapeutic effects in patients with different molecular subtypes of breast cancer. The resistance to chemotherapeutic drugs is the primary problem that affects and limits the clinical application of numerous chemotherapeutic drugs including ADR. It is also the major cause of the failure to treat patients with radiotherapy and chemotherapy at late stages, causing recurrence and even death to breast cancer patients, so the drug resistance in patients serves as the main barrier of successful radiotherapy and chemotherapy[18-20]. Additionally, studying the mechanism of multi-drug resistance in breast cancer will provide novel targets and approaches for the clinical treatment of this disease. MiR-7, an important molecule regulating the drug resistance of breast cancer cells, plays a vital role in the treatment of breast cancer. Shen et al[21] have suggested that each kind of miRNA is different in the expression in various malignant tumors, and the difference in miR-7 expression is extremely significant between breast cancer and brain glioma[22]. According to Perez et al[23], the incidence and development of breast cancer is closely related to some gene pathways, and the regulation of the expression of EGFR/PI3K signaling pathway by miRNAs has been emphasized in recent years[24]. In this study, after transfection, miR-7 sequences were expressed in MCF-10A, MCF-7 and MCF-7/

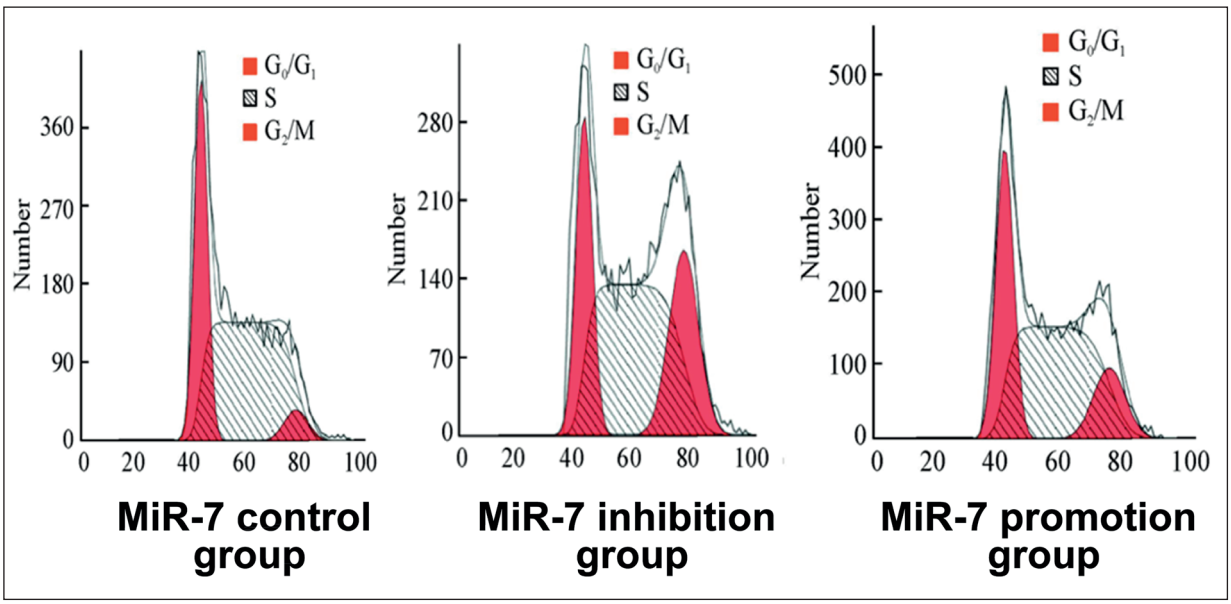

Figure 7. Relative apoptosis ability of MCF-7/ADR cells. Note: After ADR action, the cell apoptosis ability is markedly weakened in miR-7 inhibition group, but notably enhanced in miR-7 promotion group.

ADR cells. The results revealed that the expression level of miR-7 was substantially lowered in MCF-7 and MCF7/ADR cells ($p<0.05$), and compared with that in MCF-7, the decrease was more evident in MCF7/ADR ($p<0.05$). The study of Latreille et al[25] demonstrated that up-regulating the expression of miR-7 can raise the sensitivity of MCF-7/ADR to ADR, and the possible mechanism is to regulate ABCC10. In the present study, the MCF-7/ADR model of drug resistance in breast cancer was established to detect the expression of miR-7 in MCF-7/ADR. According to the results, compared with that in miR-7 control group, the expression of miR-7 in miR-7 promotion group was notably raised ($p<0.05$), proving that the sensitivity of MCF-7/ADR cells to ADR was enhanced, while it was obviously lowered in miR-7 inhibition group ($p<0.05$), suggesting that the inhibition of miR-7 can weaken the sensitivity to ADR. Additionally, when miR-7 expression was raised, the levels of EGFR and PI3K were decreased, whereas they were elevated once miR-7 expression was decreased. The mRNA and protein expressions of EGFR and PI3K were detected in the three groups as well, and the results showed that compared with those in miR-7 control group, the mRNA and protein expression levels of EGFR and PI3K were elevated in miR-7 inhibition group ($p<0.05$), while they were lowered in miR-7 promotion group ($p<0.05$). Moreover, cell proliferation and apoptosis abilities were measured in the three groups. According to the results, the proliferation ability of cells in miR-7 inhibition group was markedly higher than that in miR-7 control group ($p<0.05$), and it was poorer in miR-7 promotion group than that in miR-7 control group ($p<0.05$), suggesting that miR-7 can relatively significantly weaken the proliferation ability of drug-resistant cells. Compared with that in miR-7 control group, the apoptosis ability of cells was markedly weakened in miR-7 inhibition group ($p<0.05$), but it was evidently enhanced in miR-7 promotion group ($p<0.05$), which was consistent with the findings in the flow cytometry. Thus, the inhibition of miR-7 can alleviate the apoptosis of drug-resistant cells. Latreille et al[25] have found that miR-7 can effectively promote the drug resistance of breast cancer cells and cell apoptosis, and with the target gene of ERBB2, it can suppress the drug resistance via reducing P13KCA and Akt molecules in the PI3K/Akt signaling pathway[26]. Yan et al[27] have proven that in addition to reducing the related molecule Bcl-2 to repress drug resistance, miR-7 can target ERBB2 to down-regulate the PI3K/Akt signaling pathway, thereby inhibiting the drug resistance of breast cancer cells[27,28]. Therefore, the inhibition of the EGFR/PI3K by miR-7 can suppress drug resistance.

Conclusions

We found that miR-7 plays an important role in the resistance of breast cancer cells to ADR, and over-expressing miR-7 can enhance the sensitivity of the breast cancer cells to the chemotherapy drug ADR through inhibiting the EGFR/PI3K signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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